The amendments and new claims are fully supported by the specification and do not introduce new matter. Specifically, the amendment to claims 16, 25, 27, 34 and 44 to recite that the level of BAG-1 protein is determined using a BAG-1 specific antibody is supported, for example, at page 12, lines 5-8 and by the claims as filed. New claim 49, which recites that the disease-free survival is distant metastasis-free survival, is supported, for example, at page 35, lines 5-7 and at page 7, lines 16-24. New claims 51-56, which recite that the BAG-1 protein level is determined using immunohistochemistry, are supported, for example, at page 13, lines 21-23, and page 32, lines 11-14. The remaining amendments and new claims specify the invention with more particularity and are supported throughout the specification. A marked-up copy of the amended and newly added claims is provided in Appendix A attached hereto.

Because the amendments and new claims are fully supported by the specification and do not introduce new matter, entry thereof is respectfully requested.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 6-8, 11-14, 16, 18-27, 29-37 and 44 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking adequate enablement. The Action acknowledges that the specification enables:

> ...detecting increased BAG-1 expression in breast cancer tissue, detected using immunohistochemical staining, as an indicator

> of increased overall survival or distant metastasis free survival in Stage I or Stage II breast cancer patients...

However, the Action alleges that the specification is not enabling for other embodiments. This rejection is respectfully traversed for the reasons that follow.

Method of determining BAG-1 protein expression

The Action notes that the claims are directed to methods of "detecting BAG protein or mRNA expression" (emphasis added). While Applicant maintains that the specification enables practice of the method by detecting either BAG protein or BAG mRNA expression, the claims as amended now specify methods of "determining, using a BAG-1 specific antibody, the level of BAG-1 protein expression."

The Action asserts that "all of the teachings in the instant specification...are drawn to immunohistochemical detection of BAG-1..." (emphasis added). In contrast, the specification teaches several alternative methods of detecting BAG-1 protein expression using a BAG-1 specific antibody. For example, the specification teaches that BAG-1 protein levels can alternatively be determined using Western Blot analysis, immunoprecipitation, immunoassays such as ELISA and immuno-PCR, and the like (page 13, lines 21-29; page 14, lines 4-19).

As corroboration that methods other than immunohistochemical detection can be used to determine the level

of BAG-1 expression, attached as Exhibit A is a publication by Shindoh et al., Oral Oncology 36:444-449 (2000). Exhibit A describes a comparison of immunohistochemical staining for BAG-1 and Western blot analysis of BAG-1 expression in oral squamous cell carcinoma biopsies. The results from immunohistochemical analysis are shown in Table 1, with the results from Western blot analysis for cases 8, 2, 5, 6, 11, 17, 18, 19 and 9 (lanes 1-9, respectively) shown in Figure 2. In each of the nine biopsies examined by both methods, the level of BAG-1 expression determined by immunohistochemistry ("high" or "low") correlated with the relative steady state level of BAG-1 (above or below 1.0, respectively) determined by Western blot analysis. The authors concluded (last sentence, page 446) that:

> Western blot analyses corroborated the BAG-1 immunostaining results (Fig. 2).

Therefore, Exhibit A corroborates the teachings in the specification that the level of BAG-1 protein expression can be determined using various methods of detecting BAG-1 protein expression using a BAG-1 specific antibody.

Detection of BAG-1 protein expression in body fluid

The Action notes that BAG-1 is described as a cytosolic or nuclear protein, and states that:

> Detection of a cytosolic protein or a nuclear protein does not support the suggestion that the protein will also be

> shed into body fluids, and in particular does not provide the suggestion that the protein will be shed into a body fluid in sufficient quantities to be diagnostic for breast cancer.

However, the specification teaches that:

> ...a body fluid sample, such as a lymph, blood or serum sample, or an exudate fluid sample such as the cancerous organ exudate (e.g., exudate from the breast) may be collected and used as the sample to be analyzed... While BAG levels will typically be measured within the cancerous cells of a patient, levels of BAG will also be measured in a body fluid sample (e.g., serum) as a result of BAG having been secreted or otherwise released from cells (e.g., by cell rupture). (page 8, lines 9-23)

Accordingly, the specification teaches that the claimed methods can be practiced by determining the level of BAG-1 expression either in a tumor or in a body fluid, because BAG-1 can be released from cells into body fluid.

As corroboration that BAG-1 protein can be released into body fluid, attached hereto as Exhibit B is a Rule 132 Declaration by Dr. Lloyd Hutchinson. Exhibit B demonstrates that BAG-1 protein can be detected and quantitated in human urine by a competitive ELISA assay.

Based on the teachings in the specification, the skilled person would expect that the level of BAG-1 protein

released into the urine, or into other body fluid, would be correlated with the level in the breast cancer tissue. Therefore, Exhibit B corroborates the teachings in the specification that the level of BAG-1 protein expression can be determined in either a tumor or a body fluid.

Correlation of BAG-1 expression with various parameters

The Action acknowledges that the specification is enabling for methods of detecting increased BAG-1 expression in stage I or II breast cancer as an indicator of increased overall survival (OS) or distant metastasis-free survival (DMFS). However, the Action alleges that the specification is not enabling for "detection of an increase or decrease in BAG-1 gene expression...further as an indicator of prognosis, risk of recurrence, risk of metastasis or monitoring treatment effectiveness" (page 3, lines 1-3).

All pending claims refer to a positive correlation between BAG-1 protein levels and outcome in stage I and stage II breast cancer. All pending claims recite parameters that are well known to be directly correlated with OS and DMFS, namely disease-free survival, (lack of) tumor recurrence or spread, and (lack of) tumor metastasis. Specifically, claim 16 and its dependents recite methods of prognosis of disease-free or overall survival; claim 25 and its dependents recite methods for predicting tumor recurrence or spread; claim 27 and its dependents recite methods of screening to determine the risk of tumor metastasis or chance of survival; claim 34 and its

dependents recite methods of determining the proper course of treatment by identifying patients having higher or lower chance of survival or being more or less likely to suffer tumor recurrence or spread; and claim 44 and its dependents are directed to methods of determining risk of tumor recurrence or spread. As taught in the specification, "tumor recurrence" refers to further growth of neoplastic or cancer cells after diagnosis; "tumor spread" occurs when tumor cells disseminate locally or into distant tissues and organs, and encompasses metastasis; and "disease-free survival" refers to the lack of tumor recurrence and/or spread (page 7, lines 11-24). Accordingly, it follows that if the specification enables the claimed methods with respect to a prediction of likelihood of OS or DMFS, it also enables the claimed methods with respect to the prediction of the interrelated parameters, namely disease-free survival, likelihood of tumor recurrence or spread or metastasis.

The Action (page 4, lines 3-4) makes the observation that the "art teaches that increased levels of BAG expression do not indicate decreased risk of metastasis or recurrence for cancer." The Action cites several references in support of this assertion. Two references examined the effect of transfecting BAG-1 into non-breast cancer cell lines, namely murine melanoma cell lines (Takaoka et al.) and murine gastric cancer cell lines (Yawata et al.). However, as noted in Exhibit A,

> ...apoptosis is regulated by the balance of anti-apoptotic molecules and pro-apoptotic molecules [25-27],

> and the biological activity of these molecules may vary in a cell-type specific manner. (page 447, column 1, first paragraph of Discussion; emphasis added)

Because the function of BAG-1, an apoptotic regulatory molecule, can vary in a cell-type specific manner, it is improper to draw any conclusions relating the effect of increased levels of BAG-1 expression in irrelevant cell types to its prognostic significance in stage I and II breast cancer.

With regard to Zapata et al., the Action states that this reference "teaches that BAG-1 levels are higher in invasive breast cancers." The passage summarized in the Action actually states that "the intensity of BAG-1 immunostaining was often higher in invasive cancers compared to normal epithelium" (page 138, column 1, paragraph 3, emphasis added). Zapata et al. examined the percentage of BAG-1 positive cells and the intensity of BAG-1 staining in three types of cells, namely normal cells, carcinoma *in situ* and invasive carcinoma cells, present in each of 20 unstaged breast cancer biopsy samples (page 131, columns 1 and 2). BAG-1 expression was shown to be generally higher in cancerous cells compared with normal cells (statistically significant for *in situ* carcinoma cells vs. normal cells and a trend for invasive cancer cells vs. normal cells; see Table 3; see also Table 2 and Figure 2). However, no significant difference was observed between BAG-1 intensity in invasive cancer cells and in *in situ* carcinoma cells within the same sample (see Table 3; note that actually more *in situ* than invasive cells exhibited intensity level 3). From Zapata et al.

no conclusion can be drawn relating relative "invasiveness" of a cancer cell to level of BAG-1 expression, and certainly no conclusion can be drawn that casts doubt on the enablement of the claimed invention.

Finally, Tang et al. is described in the Action as teaching that "increased BAG-1 expression was significantly associated with shorter disease free and overall survival in all stages of breast cancer," and Table IV is specifically referenced. However, this statement cannot be attributed to the data shown in Table IV, which is the only data presented in which the four tumor stages were examined individually. Tang et al. actually states that the data shown in Table IV, with *p* values for stage I and II breast cancer and survival of between 0.162 and 0.402, was not statistically significant (page 1713, column 2, paragraph 2). It should be noted that significant association of two variables is generally considered to be reflected by $p \leq 0.05$ (see, for example, the specification at page 35, lines 7-9). Data that is not statistically significant, and is further characterized by the authors as only "preliminary" (see abstract) cannot cast doubt on the teachings in the specification.

Furthermore, even if some conclusion were to be drawn from Tang et al. about BAG-1 levels and survival, the Action has already acknowledged that the specification enables methods of detecting increased BAG-1 expression in stage I or II breast cancer as an indicator of increased overall survival (OS) or distant metastasis-free survival (DMFS). Tang et al. does not

address the parameters that are in question in the Action, namely the correlation between BAG-1 expression and risk of tumor recurrence or spread or metastasis in stage I or stage II breast cancer. Thus, Tang et al. should not be used in support of an assertion of lack of enablement of claims that recite these parameters.

In view of the above remarks and amendments, it is respectfully submitted that the specification adequately enables the claims, and removal of the rejection under 35 U.S.C. § 112, first paragraph, is respectfully requested.

CONCLUSION

In light of the Amendments and Remarks herein, Applicant submits that the claims are now in condition for allowance and respectfully requests a notice to this effect. Should the Examiner have any questions, she is invited to call Cathryn Campbell or the undersigned agent.

Respectfully submitted,

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APPENDIX A

[6. (Canceled) The method of claim 16, wherein said level of BAG-1 expression is determined by measuring the amount of the BAG-1 mRNA transcript or BAG-1 protein.]

[7. (Canceled) The method of claim 6, wherein said measuring the amount of BAG-1 protein is with an agent that binds BAG-1 protein.]

[8. (Canceled) The method of claim 7, wherein said agent is an antibody specific for the BAG-1 protein.]

11. (Amended) The method of claim **16** **[6]**, wherein said level of BAG-1 expression is determined by measuring the amount of BAG-1 protein product using an immunoassay.

16. (Amended) A method for prognosis of disease-free or overall survival of an individual having a **breast** cancer tumor, comprising determining**, using a BAG-1 specific antibody,** the level of BAG-1 **protein** expression in a sample of said tumor or a body fluid during stage I or stage II of said cancer, wherein a high level of BAG-1 expression correlates positively with disease-free or overall survival**[, wherein said cancer is breast cancer]**.

[18. (Canceled) The method of claim 16, wherein said level of BAG-1 expression is determined by measuring the level of mRNA encoding BAG-1.]

[19. (Canceled) The method of claim 16, wherein said level of BAG-1 expression is determined by measuring BAG-1 protein levels.]

25. (Amended) A method for predicting the risk of tumor recurrence or spread in an individual having a **breast** cancer tumor, comprising determining**, using a BAG-1 specific antibody, the level of BAG-1 protein expression** **[whether BAG-1 protein is produced]** in a sample of said tumor or body fluid from said individual **during stage I or stage II of said cancer, wherein a high level of BAG-1 expression correlates** **[such a production correlating]** negatively with **[a likelihood of]** tumor recurrence or spread**[, wherein said cancer is stage I or stage II breast cancer]**.

27. (Amended) A method for screening a **breast** cancer patient to determine the risk of tumor metastasis **or chance of survival**, said method comprising:

(a) determining**, using a BAG-1 specific antibody,** the level of **[amplification or]** expression of BAG-1 **protein** in a cancerous tissue sample or a body fluid sample from said patient during stage I or stage II of said cancer; and

(b) classifying a patient having high levels of **[amplification or]** expression of BAG-1 **protein**, relative to a reference level, as being less likely to suffer tumor metastasis or having **[a] an** increased chance of **survival[, wherein said cancer is breast cancer].**

[29. (Canceled) The method of claim 27, wherein BAG-1 amplification is measured with a probe specific for BAG-1.]

[30. (Canceled) The method of claim 27, wherein gene expression is determined by measuring the amount of BAG-1 mRNA transcription.]

[31. (Canceled) The method of claim 27, wherein gene expression is determined by measuring the amount of BAG-1 protein.]

32. (Amended) The method of claim **27 [31]**, wherein the amount of BAG-1 protein is measured using an immunoassay.

34. (Amended) A method for determining the proper course of treatment for a patient suffering from **breast** cancer, said method comprising:

(a) determining**, using a BAG-1 specific antibody,** the level of BAG-1 **protein** expression in a cancerous tissue sample or body fluid from said patient during stage I or stage II of said cancer;

(b) identifying a first group of patients having low levels of BAG-1 expression, which first group of patients may require treatment proper for patients having a lesser chance of survival or **being more likely to suffer [decreased time to]** tumor recurrence or spread; and

(c) identifying a second group of patients having high levels of BAG-1 expression, which second group of patients may require treatment proper for patients having a greater chance of survival and being less likely to suffer tumor recurrence or spread**[**,

wherein said cancer is breast cancer].

[35. (Canceled) The method of claim 34, wherein said level of BAG-1 expression is determined by measuring the amount of BAG-1 mRNA transcript or BAG protein.]

44. A method for determining **risk of tumor recurrence or spread [a prognosis]** in a patient suffering from **breast** cancer, said method comprising:

(a) determining**, using a BAG-1 specific antibody,** the level of expression of BAG-1 **protein** in **a** cancerous **tissue**

[tissues] of a patient during stage I or stage II of said cancer; and

(b) classifying said patient as belonging either to a first group of patients having high levels of expression of BAG-1, or a second group of patients having low levels of expression of BAG-1,

wherein said first group has a lower likelihood of tumor recurrence or spread than said second group**[, and wherein said cancer is breast cancer]**.

45. (New) The method of claim 16, wherein said level of BAG-1 expression is determined by measuring the level of BAG-1 protein in a sample of breast tumor tissue.

46. (New) The method of claim 25, wherein said level of BAG-1 expression is determined by measuring the level of BAG-1 protein in a sample of breast tumor tissue.

47. (New) The method of claim 27, wherein said level of BAG-1 expression is determined by measuring the level of BAG-1 protein in a sample of breast tumor tissue.

48. (New) The method of claim 34, wherein said level of BAG-1 expression is determined by measuring the level of BAG-1 protein in a sample of breast tumor tissue.

49. (New) The method of claim 16, wherein said disease-free survival is distant metastasis-free survival.

50. (New) The method of claim 49, wherein said level of BAG-1 expression is determined by measuring the level of BAG-1 protein in a sample of breast tumor tissue.

51. (New) The method of claim 16, wherein said level of BAG-1 expression is determined by immunohistochemistry.

52. (New) The method of claim 50, wherein said level of BAG-1 expression is determined by immunohistochemistry.

53. (New) The method of claim 25, wherein said level of BAG-1 expression is determined by immunohistochemistry.

54. (New) The method of claim 27, wherein said level of BAG-1 expression is determined by immunohistochemistry.

55. (New) The method of claim 34, wherein said level of BAG-1 expression is determined by immunohistochemistry.

56. (New) The method of claim 44, wherein said level of BAG-1 expression is determined by immunohistochemistry.

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BAG-1 expression correlates highly with the malignant potential in early lesions (T1 and T2) of oral squamous cell carcinoma

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Abstract

BAG-1 is a Bcl-2-binding protein that functions as an anti-apoptotic molecule. In this report we show a possible correlation between BAG-1 expression levels and the probability of oral squamous cell carcinoma (SCC) progression. We investigated BAG-1 expression levels in 22 patients diagnosed with early lesions (T1 and T2) of oral SCCs using immunohistochemistry and western blotting. High steady-state levels of BAG-1 were detected in 13 out of 22 cases (59%). High BAG-1 expression was observed more frequently in cases with nodal metastasis (89%) than in those without nodal metastasis (38%) ($P < 0.03$), suggesting that BAG-1 expression levels may correlate with the pathological stage of oral SCCs. Furthermore, BAG-1 expression levels correlated with the WHO grade, i.e. 45% in grade-I cases as opposed to 72% in grade-II cases ($P < 0.02$). These data suggest that an analysis of BAG-1 expression may be useful in establishing a prognosis for patients with oral SCCs, and especially in predicting the metastatic potential of SSCs.

Keywords: BAG-1; Oral squamous cell carcinoma; T1 and T2

1. Introduction

Invasive and metastatic potential of cancer cells is likely to affect prognosis in cancer patients. To improve the prognoses, it is crucial to predict this potential prior to the beginning of cancer therapies. To date, various factors have been found to be involved in regulating the metastatic potential of cancer cells. It has been shown, for example, that steady-state levels of proteinases such as matrix metalloproteinases are elevated in invasive human squamous cell carcinomas (SCCs) and correlate strongly with cancer metastasis [1–4].

Steady-state levels of urokinase-type plasminogen activator, responsible for degradation of extracellular matrices, are elevated in a variety of invasive carcinomas [5–7]. In addition, cell adhesion molecules are thought to be involved in metastasis. Disappearance of the cell adhesion molecule E-cadherin causes disruption of cell–cell contacts that induce tumor cell invasion and metastasis [6,8,9]. Motility was also shown to be associated with metastasis [10–12]. Hepatocyte growth factor (HGF)/scatter factor was initially identified as a motility-stimulating factor in human MRC5 fibroblasts [13], and shown to promote migration and invasion of malignant epithelial cells that resulted in metastatic phenotype of various cancer cells in tissue culture [14–16]. However, the mechanism of metastasis is very complicated [17,18] and there may be other factors involved in cancer cell metastasis in oral SCCs in addition to the adhesion molecules, extracellular matrix-degrading enzymes and motility-stimulating factors.

BAG-1 is a novel Bcl-2-binding protein that can prolong cell survival in cooperation with Bcl-2 [19]. Recently, we have shown that overexpression of BAG-1 leads to prolonged cell survival of malignant melanomas and promotes their metastasis to lung [20]. BAG-1 is expressed ubiquitously [19] and seems to protect a wide variety of cancer cell types from apoptosis and promote their metastatic potential. This observation has prompted us to investigate whether BAG-1 expression correlates with the metastatic potential of other cancer cells. In the present study we have focused on the correlation

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between BAG-1 expression and the clinico-pathological malignancies of oral SCCs.

2. Materials and methods

2.1. Materials and clinical investigation

Twenty-two patients diagnosed with oral SCC were investigated. Fresh biopsy specimens from oral SCCs were collected at the Department of Oral Surgery, Hokkaido University Dental Hospital, between January 1996 and June 1997. Clinical features of the samples are shown in Table 1. Clinical staging of the disease was determined according to the International Union Against Cancer Categories (UICC) criteria (1987) [21].

2.2. Histopathological examination

Specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin and examined histopathologically. Histopathological estimation of cancer malignancies was undertaken using the World Health Organization (WHO) classification (Table 1) [22].

2.3. Immunohistochemical studies

Snap-frozen specimens were sectioned at 5-μm with a Cryostat (Leizz, Germany) and fixed in cold acetone for 10 min. They were pre-incubated for 30 min with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), and then incubated for an additional hour with a primary monoclonal antibody against human BAG-1 [19]. Bag-1 negative and positive cell lines, were used as controls. MKN-74, a gastric carcinoma cell line, was used as the negative control and MKN-74 transfected with *bag-1* expression vector was used as the positive control. A biotin-conjugated anti-mouse secondary antibody was then applied, then the avidin–biotin–peroxidase reaction followed by Liquid diaminobenzidine (DAB) substrate chromogen system (DAKO, Carpinteria, CA, USA) to visualize the reaction products was carried out. Cases which showed stronger signals for BAG-1 than the adjacent intact normal epithelium were diagnosed as high BAG-1 expression tumors. On the other hand, low BAG-1 expression tumors were cases which showed little signal for BAG-1 and no remarkable difference between tumor cells and adjacent intact epithelium. The immunohistochemical intensity of examined cases was assessed by two different pathologists.

Table 1

Case No.	Age	Gender	Primary site	T	N	Subsequent metastasis	WHO	BAG-1 expression
1	47	M	Tongue	2	0	+	I	High
2	70	M	Tongue	2	1		II	High
3	58	M	Floor of mouth	2	2		II	High
4	69	M	Tongue	2	0	+	II	High
5	52	M	Gingiva	2	2		II	Low
6	66	M	Tongue	2	2		II	High
7	54	M	Maxilla	2	2		II	High
8	74	M	Gingiva	2	0	+	I	High
9	81	F	Tongue	2	1		II	High
10	52	M	Tongue	2	0		I	High
11	69	M	Buccal mucosa	2	0		I	Low
12	76	F	Tongue	1	0		II	High
13	85	M	Gingiva	2	0		I	Low
14	68	F	Maxilla	2	0		II	High
15	28	F	Tongue	2	0		II	Low
16	53	M	Gingiva	1	0		I	High
17	67	F	Gingiva	2	0		I	High
18	67	M	Tongue	1	0		I	Low
19	67	F	Gingiva	2	0		H	Low
20	72	F	Tongue	2	0		I	Low
21	33	M	Buccal mucosa	1	0		I	Low
22	63	F	Tongue	1	0		I	Low

2.4. Western blot analysis

Tumor tissues were snap-frozen in liquid nitrogen and stored at −80°C until needed. Approximately 50 mg of each tissue sample was lysed in 100 ml of the radio-immuno-protein-assay (RIPA) buffer containing 100 mM NaCl, 2 mM EDTA, 10 mM sodium orthovanadate, 1 mM phenylmethane sulfonyl fluoride (PMSF), 1% NP-40 and 50 mM Tris (pH 7.2). Protein concentrations of the lysates were determined with a Bradford-based protein assay kit (BioRad, Richmond, CA, USA) and each lysate (80 μg/lane) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by an electrophoretic transfer onto Immobilon membranes (Millipore, Bedford, MA, USA). Blots were incubated with a blocking buffer containing 3% BSA, 10 mM Tris (pH 8.2), 140 mM NaCl, and 0.01% NaN_3. They were then incubated for 2 h with an anti-human BAG-1 monoclonal antibody [19] diluted in a washing buffer containing 150 mM NaCl, 10 mM Tris (pH 7.5) and 0.01% Tween 20, and supplemented with 3% fetal bovine serum. The blots were developed by a standard enhanced chemiluminescence method (Amersham, Buckinghamshire, UK). The same blots were subsequently incubated with 0.5 mg/ml of an anti-actin monoclonal antibody (Sigma, Osaka, Japan) to verify the amount of loading protein in each lane.

2.5. Estimation of BAG-1 expression level

The density of each sample was measured by the densitometer (IBAS, Leizz, Germany). The relative expression levels were calculated as BAG-1/actin ratio.

2.6. Statistical analysis

Independence of clinical parameters and histological features of BAG-1 expression was examined with the Fisher test.

3. Results

Table 1 shows the clinico-pathological findings in 22 oral SCC patients who were 28–85 years old. Primary regions included the tongue (11 cases), cheek (two cases), floor of the mouth (one case), gingiva (six cases) and maxilla (two cases). Clinical staging of patients was undertaken utilizing the UICC criteria (1987). Among 22 patients, five were classified as T1, and 17 as T2. Sixteen cases were classified as N0, two as N1 and four as N2 in primary clinical examinations. Subsequent metastasis was found in three of the 16 N0 cases in the follow-up period that lasted between six and 24 months (Table 1). The WHO classification was applied to assess histological malignancy. There were 11 grade-I cases and 11 grade-II cases. BAG-1 protein expression was investigated in all specimens. A strong BAG-1 reaction product was detected in 13 of the 22 oral SCCs (59%) by immunohistochemistry (Fig. 1). The normal epithelium showed no obvious reaction products for BAG-1 antibody (Fig. 1A). We defined "high" BAG-1 expression as tumor cells immunostaining which showed stronger signals than the adjacent intact normal epithelium (Fig. 1B). BAG-1 protein was seen in the cytoplasm of cancer cells invading into the connective tissues (Fig. 1C). Among the cases with "low" BAG-1 immunostaining the levels of BAG-1 in cancer cells were similar to those observed in normal epithelial cells (Fig. 1D). MKN-74 cells showed no obvious signal for BAG-1, while *bag-1* transfected MKN-74 cells were positive for BAG-1 antibody (data not shown). Western blot analyses corraborated the BAG-1 immunostaining results (Fig. 2).

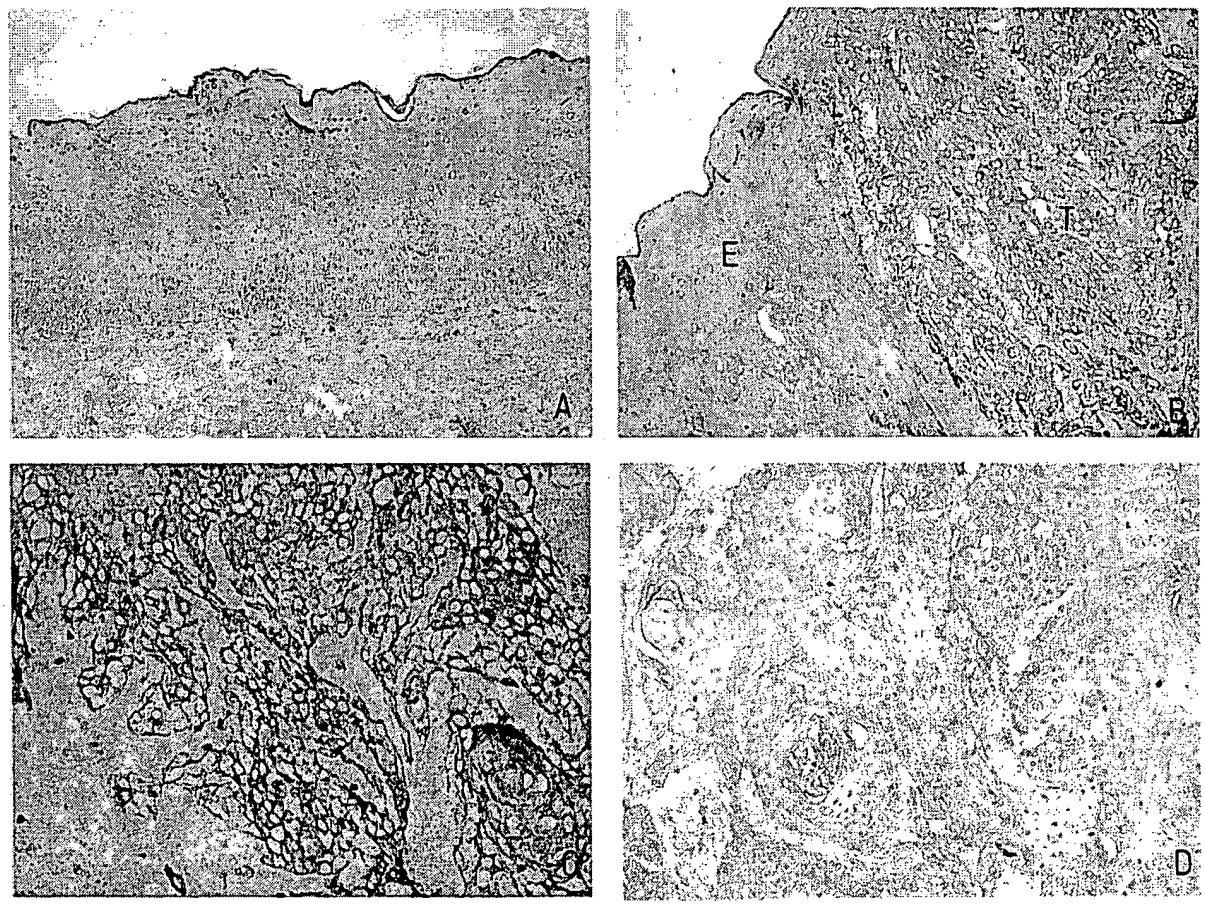

Fig. 1. Immunohistochemical analysis of BAG-1 expression in cells. (A) BAG-1 expression in normal epithelium. No remarkable reaction products were seen in the epithelial layer (×50). (B) High steady-state levels of BAG-1 were found in case 6. An intense signal for BAG-1 was observed in invading tumor cells (T), whereas little signal was seen in the adjacent intact epithelium (E) (×50). (C) Same cells as in (D) at a higher magnification. BAG-1 was detected in the cytoplasm of cancer cells invading into the connective tissue (×100). (D) Low BAG-1 expression in case 18. Little BAG-1 signal was observed in cancer cells (×100).

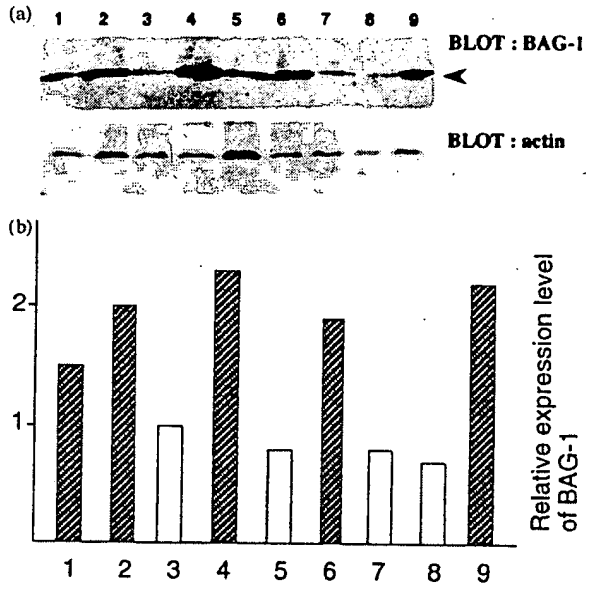

Fig. 2. Western blotting detection of BAG-1. (a) Total cell lysates from tissue samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/immunoblotting with an anti-BAG-1 monoclonal antibody. The membrane was reprobed with an anti-actin antibody to verify equal loading of protein. Western blot analysis was performed by a standard enhanced chemiluminescence (ECL) method (Amersham). (b) Relative steady-state levels of BAG-1 and actin were measured with a densitometer. Lane 1, case 8; Lane 2, case 2; Lane 3, case 5; Lane 4, case 6; Lane 5, case 11; Lane 6, case 17; Lane 7, case 18; Lane 8, case 19; Lane 9, case 9.

High steady-state levels of BAG-1 were found in two of five T1 and 11 of 17 T2 samples (Table 1). High BAG-1 expression was found more frequently in the N1/N2 stage cases and in cases characterized by subsequent metastases (89%) than in the N0 cases (38%) ($P < 0.03$). When tumors were classified according to histological grade, high BAG-1 expression was seen in five of 11 (45%) grade-I cases and in eight of 11 (73%) grade-II cases (Table 1) ($P < 0.02$).

4. Discussion

There is ample evidence showing a correlation between the expression levels of anti-apoptotic molecules and prognosis in cancer patients or the clinical aggressiveness of malignant cells [23,24]. However, since apoptosis is regulated by the balance of anti-apoptotic molecules and pro-apoptotic molecules [25–27], and the biological activity of these molecules may vary in a cell type-specific manner. Thus, detection of a single apoptosis-related molecule may, in general, not be sufficient to evaluate the relative apoptotic resistance of cancer cells.

BAG-1 is a novel multifunctional protein [19] with anti-apoptotic potential. BAG-1 has been shown to bind to Hsc70 and probably helps to target this molecular chaperone to several proteins involved in cell growth regulation. Among the proteins whose functions are enhanced by BAG-1 are the anti-apoptotic protein Bcl-2 [19,28], the protein kinase Raf-1 [28,29], and the growth factor receptors PDGF-R (platelet derived growth factor receptor) and HGFR (hepatocyte growth factor receptor) [30].

Recently, we have shown that pulmonary metastasis of malignant melanoma cells in animal models is promoted by overexpression of BAG-1 [20]. This implies that BAG-1 expression may contribute to the metastatic potential of cancer cells, and thus that the BAG-1 molecule may be a useful marker for predicting the micrometastatic disease in patients with some types of solid tumors.

In the present study we analyzed BAG-1 expression in cases with early lesions of oral SCCs (T1 and T2), and determined whether the expression level may be a factor useful in predicting their metastatic potential. We eliminated the T3/T4 cases from a subsequent analysis because we observed that they tend to metastasize whether they express metastasis-related gene products or not [31]. Our data show that BAG-1 was highly expressed in 13 (59%) of 22 SCC cases. There were no significant differences in the steady-state levels of BAG-1 due to gender or the age of the patients, or to primary tumor sites (Table 1). Neither was there a correlation between tumor sizes and BAG-1 expression levels (Table 1). However, high steady-state levels of BAG-1 were found more frequently in cases of primary metastasis or subsequent metastasis (eight of nine cases) than in cases without metastasis (five of 13 cases) ($P < 0.03$). According to a histological classification, high BAG-1 expression was found in five of 11 (45%) grade-I cases and eight of 11 (73%) grade-II cases (Table 1), suggesting that in histologically more aggressive cancer cells, the steady-state levels of BAG-1 may be higher than in less aggressive malignant cancer cells. These results are consistent with animal data that overexpression of BAG-1 promotes pulmonary metastasis of malignant melanoma cells [20], and suggest that BAG-1 protein levels are a crucial factor associated with the metastatic potential of oral SCCs.

The correlation between the high steady-state levels of BAG-1 and the metastatic potential of oral SCCs may have many explanations. For example, the anti-apoptotic function of the protein could contribute to metastasis by allowing epithelial cells to survive in a detached state, after losing its interaction with the extracellular matrix [32,33]. However, as mentioned above, BAG-1 has divergent functions other than its anti-apoptotic. Recently, BAG-1 has been shown to interact with the hepatocyte growth factor (HGF)/scatter factor receptor (HGFR) and enhancing some signals transduced through HGFR [30] Since HGF was initially identified as a motility-stimulating factor in human MRC5 fibroblasts [13], high steady-state levels of BAG-1 could be associated with an enhancement of the migration ability

of cancer cells, resulting in an increase in their metastatic potential. In addition, BAG-1 has been shown to stimulate the recycling of Hsc70 by accelerating ADP release and thus increasing the chaperone activity of Hsc70 [29,34]. Although the biological significance of this action remains to be clarified, BAG-1 may play a key role in the folding, translocation and degradation of proteins, and this activity may be associated with an attachment or detachment of cancer cells as well as its ability to survive in a foreign environment. Thus, a variety of activities of BAG-1 may be involved in the enhancement of the metastatic potential of SCC cells. Although the precise function of BAG-1 in association with the metastatic potential of cancer cells remains unknown, our data lease the possibility that BAG-1 may be a predictor of the metastatic potential of oral SCCs, a hypotheses which deserves testing in future studies involving larger groups of patients.

Acknowledgements

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References

[1] Alessandro R, Minafra S, Pucci MI, Onisto M, Garbisa S, Melchiori A, Tetlow L, Woolley DE. Metalloproteinase and TIMP expression by the human breast carcinoma cell line 8701-BC. Int J Cancer 1993;55:250–5.

[2] Bernhard EJ, Gruber SB, Muschel RJ. Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. Proc Natl Acad Sci USA 1994;91:4293–7.

[3] Juarez J, Clayman G, Nakajima M, Tanabe KK, Saya H, Nicolson GL, Boyd D. Role and regulation of expression of 92-kDa type-IV collagenase (MMP-9) in 2 invasive squamous-cell-carcinoma cell lines of the oral cavity. Int J Cancer 1993;55:10–18.

[4] Shindoh M, Higashino F, Kaya M, Yasuda M, Funaoka K, Hanzawa M, Hida K, Kohgo T, Amemiya A, Yoshida K, Fujinaga K. Correlated expression of matrix metalloproteinases and ets family transcription factor E1A-F in invasive oral squamous-cell-carcinoma-derived cell lines. Am J Pathol 1996;148:693–700.

[5] Liotta LA, Thorgeirsson UP, Gatbisa S. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 1991;64:327–36.

[6] Portella G, Liddell J, Crombie R, Haddow S, Clarke M, Stoler AB, Balmain A. Molecular mechanisms of invasion and metastasis during mouse skin tumour progression. Invasion Metastasis 1994;14:7–16.

[7] Duggan C, Maguire T, McDermott E, O'Higgins N, Fennelly JJ, Duffy MJ. Urokinase plasminogen activator and urokinase plasminogen activator receptor in breast cancer. Int J Cancer 1995;61:597–600.

[8] Behrens J. The role of cell adhesion molecules in cancer invasion and metastasis. Breast Cancer Res Treat 1993;24:175–84.

[9] Kinsella AR, Green B, Lepts GC, Hill CL, Bowie G, Taylor BA. The role of the cell–cell adhesion molecule E-cadherin in large bowel tumour cell invasion and metastasis. Br J Cancer 1993;67:904–9.

[10] Mohler JL, Partin AW, Isaacs JT, Coffey DS. Metastatic potential prediction by a visual grading system of cell motility: prospective validation in the Dunning R-3327 prostatic adenocarcinoma model. Cancer Res 1988;48:4312–7.

[11] Liotta LA, Mandler R, Murano G, Katz DA, Gordon RK, Chiang PK, Schiffmann E. Tumor cell autocrine motility factor. Proc Natl Acad Sci USA 1986;83:3302–6.

[12] Partin AW, Schoeniger JS, Mohler JL, Coffey DS. Fourier analysis of cell motility: correlation of motility with metastatic potential. Proc Natl Acad Sci USA 1989;86:1254–8.

[13] Stoker M, Gherardi E, Perryman M, Gray J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature 1987;327:239–42.

[14] Matsumoto K, Matsumoto K, Nakamura T, Kramer RH. Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125FAK) and promotes migration and invasion by oral squamous cell carcinoma cells. J Biol Chem 1994;269:31 807–1 813.

[15] Bellusci S, Moens G, Gaudino G, Comoglio P, Nakamura T, Thiery JP, Jouanneau J. Creation of an hepatocyte growth factor/scatter factor autocrine loop in carcinoma cells induces invasive properties associated with increased tumorigenicity. Oncogene 1994;9:1091–9.

[16] Rosen EM, Knesel J, Goldberg ID, Jin L, Bhargava M, Joseph A, Zitnik R, Wines J, Kelley M, Rockwell S. Scatter factor modulates the metastatic phenotype of the EMT6 mouse mammary tumor. Int J Cancer 1994;57:706–14.

[17] Fidler IJ. Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. Cancer Res 1990;50:6130–8.

[18] Nicolson GL. Metastatic tumor cell interactions with endothelium, basement membrane and tissue. Curr Opin Cell Biol 1989;1:1009–19.

[19] Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, Reed JC. Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell 1995;80:279–84.

[20] Takaoka A, Adachi M, Okuda H, Sato S, Yawata A, Hinoda Y, Takayama S, Reed JC, Imai K. Anti-cell death activity promotes pulmonary metastasis of melanoma cells. Oncogene 1997;14:2971–7.

[21] Spissel B, Beahrs O, Hermanek P, Hutter R, Schreibe O, Sobin L, Wagner G. Head and neck tumors. Berlin: Springer-Verlag, 1989.

[22] Wahi PN, Cohen B, Luthra UK, Torloni H. Histological typing of oral and oropharyngeal tumours. Geneva: World Health Organization, 1971.

[23] Kapranos N, Karaiosifidi H, Valavanis C, Kouri E, Vasilaros S. Prognostic significance of apoptosis related proteins Bcl-2 and Bax in node-negative breast cancer patients. Anticancer Res 1997;17:2499–505.

[24] Manne U, Myers RB, Moron C, Poczatek RB, Dillard S, Weiss H, Brown D, Srivastava S, Grizzle WE. Prognostic significance of Bcl-2 expression and p53 nuclear accumulation in colorectal adenocarcinoma. Int J Cancer 1997;74:346–58.

[25] Akbar AN, Borthwick NJ, Wickremasinghe RG, Panayoitidis P, Pilling D, Bofill M, Krajewski S, Reed JC, Salmon M. Interleukin-2 receptor common gamma-chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (bcl-2, bcl-xL) but not pro-apoptotic (bax, bcl-xS) gene expression. Eur J Immunol 1996;26:294–9.

[26] Lotem J, Sachs L. Regulation of bcl-2, bcl-XL and bax in the control of apoptosis by hematopoietic cytokines and dexamethasone. Cell Growth Differentiation 1995;6:647–53.

[27] Lotem J, Sachs L. Control of apoptosis in hematopoiesis and leukemia by cytokines, tumor suppressor and oncogenes. Leukemia 1996;10:925–31.

[28] Reed JC, Zha H, Aime-Sempe C, Takayama S, Wang HG. Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Adv Exp Med Biol 1996;406:99–112.

[29] Takayama S, Bimston DN, Matsuzawa S, Freeman BC, Aime-Sempe C, Xie Z, Morimoto RI, Reed JC. BAG-1 modulates the chaperone activity of Hsp70/Hsc70. EMBO J 1997;16:4887–96.
[30] Bardelli A, Longati P, Albero D, Goruppi S, Schneider C, Ponzetto C, Comoglio PM. HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death. EMBO Journal 1996;15:6205–12.
[31] Hida K, Shindoh M, Yoshida K, Kudo A, Funaoka K, Kohgo T, Fujinaga K, Totsuka Y. Expression of E1AF, an *ets*-family transcription factor, is correlated with invasive phenotype of oral squamous cell carcinoma. Oral Oncol 1997;33:426–30.
[32] Weaver VM, Fischer AH, Peterson OW, Bissell MJ. The importance of the microenvironment in breast cancer progression: recapitulation of mammary tumorigenesis using a unique human mammary epithelial cell model and a three-dimensional culture assay. Biochem Cell Biol 1996;74:833–51.
[33] Ruoslahti E. RGD and other recognition sequences for integrins. Ann Rev Cell Develop Biol 1996;12:697–715.
[34] Hohfeld J, Jentsch S. GrpE-like regulation of the Hsc70 chaperone by the anti-apoptotic protein BAG-1. EMBO J 1997;16:6209–16.1.

PATENT

Our Docket: P-LJ 3578

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
John C. Reed

Serial No: 09/350,518

Filed: July 9, 1999

For: A METHOD FOR DETERMINING THE PROGNOSIS OF CANCER PATIENTS BY MEASURING LEVELS OF BAG EXPRESSION

Examiner: J. Hunt

Group Art Unit: 1642

Commissioner for Patents
Washington, D.C. 20231

CONSIDERED RLH

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

I, Lloyd Hutchinson, declare as follows:

1) I am currently a Research Fellow in Dr. Bruce Zetter's laboratory at Children's Research Hospital in Boston, Massachusetts.

2) I received a doctorate in Molecular Virology and Immunology from McMaster University, Hamilton, Ontario, Canada, where my research focused on oncogenic viruses. Prior to my Ph.D. studies I worked for approximately two years as a research technician at the University of Guelph (Guelph, Ontario, Canada) and studied bovine leukemia virus. I have 5 years experience as a post-doctoral fellow in the field of tumor metastasis and angiogenesis.

3) My primary area of interest is in the field of cancer and, in particular, identification and analysis of proteins that contribute to tumor metastasis. Furthermore, I have considerable experience in the development of these proteins as markers for the diagnosis and prognosis of human cancers. I have co-authored eight publications and eight abstracts in the field of tumor biology and virology. The most recent and relevant are listed below:

Lin et al., "Bag-1 is a novel cytoplasmic binding partner of the membrane form of heparin-binding EGF-like growth factor," J. Biol. Chem. 276:30127-30132 (2001).

Hutchinson et al., "Thymosin beta 15 levels in urine as a prognostic marker for human prostate cancer: Assay Development and Pilot Study," Proceedings of the American Association for Cancer Research 43:639 Abstract 3176 (2002).

4) I have been informed that claims in the above-identified application have been rejected, in part, because no evidence has been presented that BAG-1 protein can be detected in body fluid.

5) I have conducted an experiment that addresses whether BAG-1 protein can be detected in human urine. Exhibit 1, attached hereto, is a copy of the experimental protocol and resulting data for a competition ELISA assay that I used to determine the amount of BAG-1 protein in human urine from a panel of urology patients having a variety of diseases, including

kidney stones, prostate cancer prior to treatment and prostate cancer post-treatment.

6) The experiment was performed as follows. A constant amount of glutathione-S-transferase-mouse-BAG-1 fusion protein (GST-msBag1) was coated onto wells of a multiwell plate. Various dilutions of GST-msBag1, or 1 ml of human urine samples, were incubated with monoclonal antibody KS10B6 (obtained from Dr. John Reed of The Burnham Institute), which recognizes both mouse and human BAG-1 protein. Following incubation, glutathione beads were added to each GST-msBag1 dilution or urine sample, and the beads pelleted. After pelleting, a constant amount of supernatant was added to individual coated wells, allowing free BAG-1 antibody to bind immobilized GST-msBag1. Following incubation, a biotin-labeled secondary antibody was added, and a chromogenic assay performed. The protocol is described in more detail on pages 1 and 2 of Exhibit 1.

7) The absorbance values for the dilutions (4000 pM to 7.81 pM), for the negative and positive controls, and for the 12 urine samples are shown in the table on page 2 of Exhibit 1. The standard curve relating the absorbance values (OD 410nm) to BAG-1 concentration (pM) is shown on page 3 of Exhibit 1. The mean absorbance values for the urine samples are shown graphically on page 4 of Exhibit 1. The absorbance values were converted to BAG-1 concentration following the procedure described on pages 5 and 6 of Exhibit 1. The calculated concentration of BAG-1 in urine from the 12 patients is shown graphically on page 6 of Exhibit 1. As shown on pages 5 and 6 of Exhibit 1, the BAG-1

concentration in urine from the 12 patients ranged from an undetectable amount (seven patients) to 114 pM (patient 171).

8) These results demonstrate that BAG-1 protein can be detected and quantitated in human urine.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Oct 1, 2002
Date

L. Hutchinson
Lloyd Hutchinson, Ph.D.

2/20/2001

Competition ELISA for BAG1 in Urine using the KS10B6 monoclonal antibody.

Note: The mouse Bag1 protein was used in these experiments.
GST-msBag1 fusion protein (4.97 mg/ml) stock

1. Coating of plate
Costar polystyrene, high binding 96 well plate
- 10ml coating buffer + 3ul Bag1 (5mg/ml stock) = 150ng/well
Add 100ul/well add incubate 2hr at 37°C.

5 mg/ml = 15mg into 10ml
= 1.5 mg/ml
.15 mg = 150 mg

2. Blocking nonspecific binding
Add 300ul/well PBS+3%BSA and incubate for 1hr at 37°C.

3. Primary antibodies: MAb KS10B6
Dilute 6ul KS10B6 BAG1 MAb into 12ml PBS+1.5% BSA (1:2000 initial dilution with a final 1:4000 dilution)

EXHIBIT 1

4. GST-BAG1 competition protein - dilution series
Create a 2-fold dilution series of the GST-MsBag1 protein in PBS (500ul/dilution) with 10 dilution steps ranging from 200000 ng/ml to 390ng/ml.

Make the following controls: Positive control = 500ul PBS + 500ul KS10B6 antibody dilution (+ glutathione beads)
Negative control = 1000ul PBS (+ glutathione beads)

5. Urine samples
1ml samples were obtained 1/24/01 and stored at –20C. Samples were thawed on ice and centrifuged at 14000rpm (21000xg) for 5min. 500ul of the supernatant was used in the next step.

6. Antibody incubation with urine sample or GST-BAG1 dilution series
Add 500ul of the 1:2000 KS10B6 antibody dilution to each GST-BAG1 protein dilution step and incubate 1hr at 37°C.

7. Addition of glutathione beads
a) Preparation of beads: Block with BSA . Mix as a 50% solution in PBS + 2%BSA
b) Add 75ul 50% bead mixture to each of the urine/dilution steps and the positive and negative controls
c) Incubate, end/end at room temperature for 1hr.
d) Centrifuge at 5000rpm (2700xg) for 2min to pellet beads
e) Use 800ul of the supernatant for the ELISA

8. Addition of Dilution series
Add 200ul of each antigen:antibody dilution to each well, and incubate 1hr at 37°C.

7. Secondary antibody:
Dilute 24ul anti-mouse IgG biotin (Vector Laboratories) in 12ml PBS + 0.75%BSA (1:500 dilution)
Add 100ul/well and incubate for 30min at 37°C.

8. Vector Laboratories ABC complex (avidin-biotin-alkaline phosphatase complex)
- prepare 30min in advance according to manufacturers directions

Add 100ul/well and incubate for 30min at 37°C.

9. Chromagen development
- prepare PNPP chromagen (Sigma) in glycine buffer

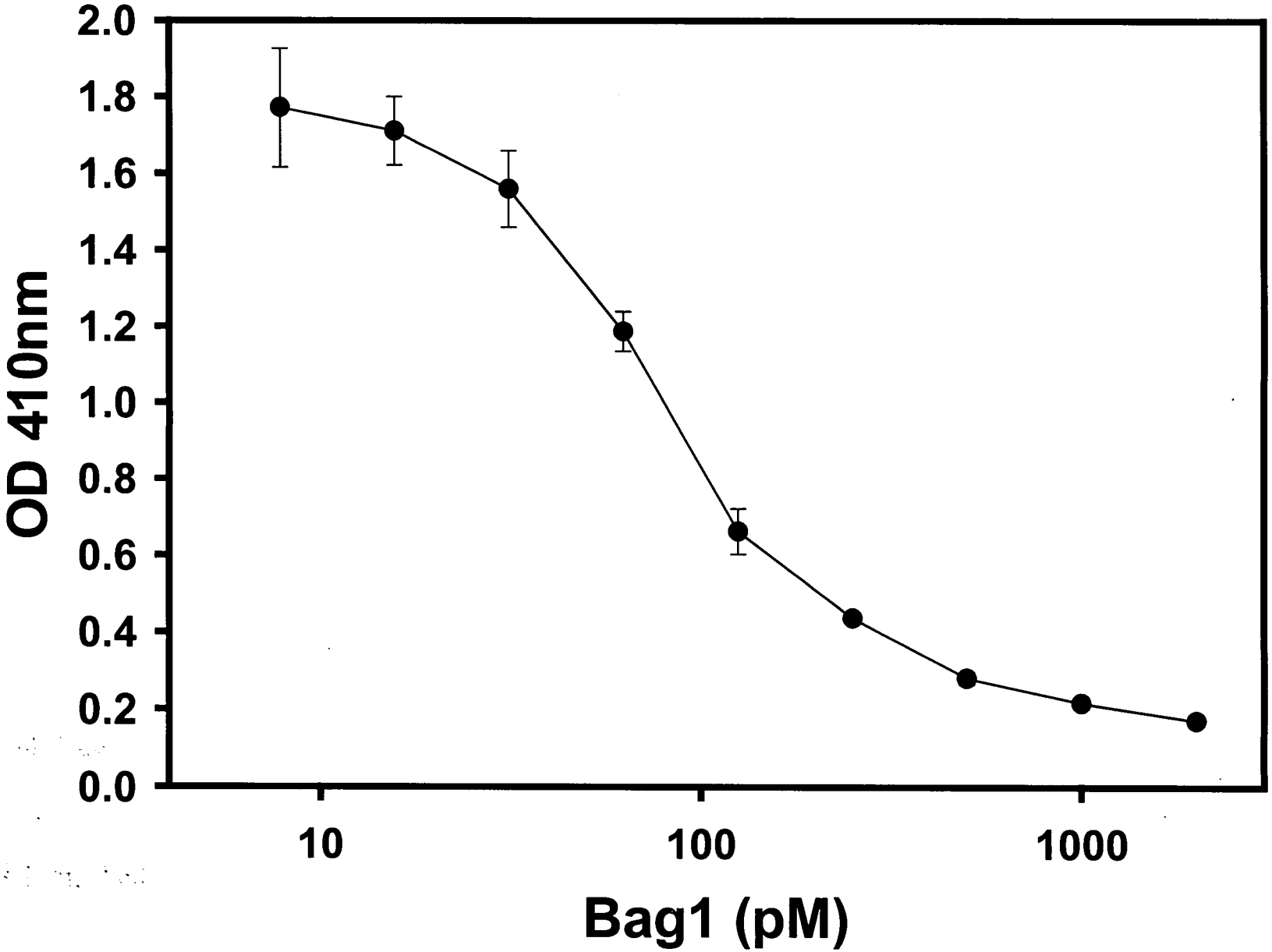
Add 100ul/well and incubate for 30min at 37°C.

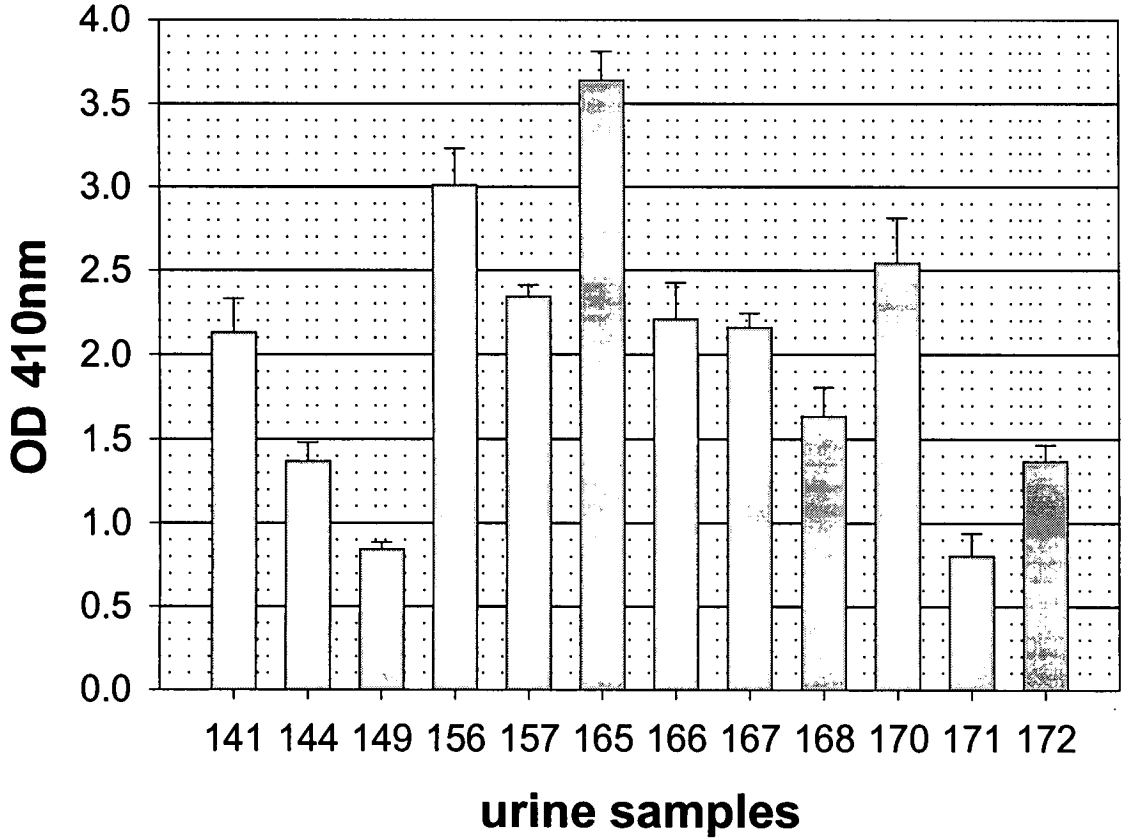
9. Results
- produced a good standard curve. Sensitivity was increased 2-fold by reducing antibody concentration to a final dilution of 1:4000
- detected Bag1 in a variety of urine samples, and although the pattern often overlapped with thymosin B15 in urine, some samples were distinct (i.e. high for TB15 and low for BAG1)

GST-MsBag1 competition standard curve #3 abd samples: 30min read

pM	4000	2000	1000	500	250	125	62.5	31.25	15.625	7.81		
ug/ml	**200000**	**100000**	**50000**	**25000**	**12500**	**6250**	**3125**	**1563**	**781**	**391**	**Neg. Control**	**Pos. Contro**
	0.2126	0.159	0.2044	0.2495	0.4248	0.6237	1.1733	1.8298	1.899	2.1642	0.09	2.0945
	0.1693	0.1536	0.1829	0.2634	0.4052	0.5379	1.0447	1.3545	1.4817	1.4844	0.0732	1.9461
	0.19	0.1881	0.2466	0.3238	0.4493	0.8217	1.2739	1.4884	1.7858	1.565	0.0842	2.0659
	0.222	0.1853	0.2313	0.2831	0.4673	0.6643	1.256	1.5625	1.6749	1.8684	0.0729	2.0139
Avg	0.198	0.172	0.216	0.280	0.437	0.662	1.187	1.559	1.710	1.771	0.080	2.030
std error	0.012	0.009	0.014	0.016	0.014	0.059	0.052	0.100	0.089	0.155	0.004	0.033
Sample#	**141**	**144**	**149**	**156**	**157**	**165**	**166**	**167**	**168**	**170**	**171**	**172**
	2.3595	1.3091	0.8357	2.4932	2.4697	3.4397	1.7043	2.1318	1.7317	2.6524	0.7271	1.5855
	1.8389	1.5478	0.741	2.7957	2.13	4	2.0513	2.3067	2.0534	3.1001	1.1939	1.3384
	1.7475	1.0684	0.8526	3.4553	2.3959	3.2662	2.3651	1.9293	1.5006	2.6218	0.7194	1.4296
	2.574	1.542	0.9444	3.2958	2.365	3.8506	2.716	2.2677	1.2669	1.8046	0.5743	1.115
Avg	2.130	1.367	0.843	3.010	2.340	3.639	2.209	2.159	1.638	2.545	0.804	1.367
std error	0.200	0.114	0.042	0.222	0.073	0.172	0.216	0.085	0.168	0.270	0.135	0.098

Bag1 Standard Curve





Conversion from urine sample OD410 to Bag1 concentration in pM:

A. Model the standard curve using SigmaPlot 4 software.

y=0.2212+1.716*exp(-0.01894*x)

y1=y-0.2212

y2=y1/1.716=exp(-0.01894*x)

y3=ln(y2)=-0.01894*x

x=y3/-0.01894

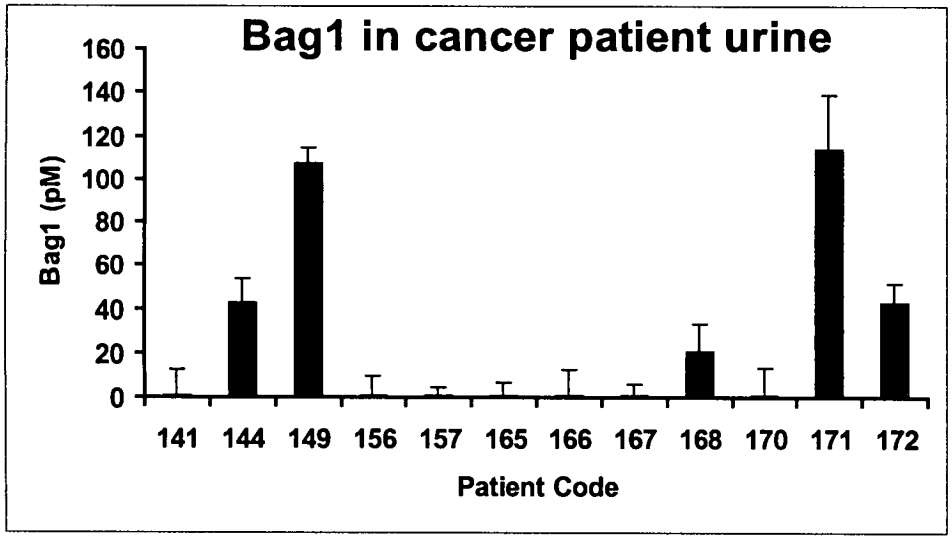
B. Calculate x to determine the Bag1 concentration in urine

Sample	Y (OD410)	y1	y2	y3	x
141	2.130	1.909	1.112	0.106	-5.621
144	1.367	1.146	0.668	-0.404	21.333
149	0.843	0.622	0.363	-1.014	53.561
156	3.010	2.789	1.625	0.486	-25.640
157	2.340	2.119	1.235	0.211	-11.136
165	3.639	3.418	1.992	0.689	-36.380
166	2.209	1.988	1.158	0.147	-7.768
167	2.159	1.938	1.129	0.121	-6.415
168	1.638	1.417	0.826	-0.191	10.110
170	2.545	2.324	1.354	0.303	-16.003
171	0.804	0.582	0.339	-1.080	57.047
172	1.367	1.146	0.668	-0.404	21.319

C. Standard error in Bag1 concentration was calculated by plugging in OD410 values +/- std error (see excel spread sheet in results above). Note: The Bag1 concentration was increased by 2-fold because our original picoMolar standard curve did not account for the GST component of GST-BAG1. Since the GST component increases MW of protein from 25 to 50kDa, the sensitivity increases by 2-fold since only have of the protein present in the assay is actually Bag1.

Sample	Avg	low	high	error
141	< 8	< 8	< 8	11.1
144	42.6659	32.652	53.7293	10.5
149	107.122	100.28	114.441	7.1
156	< 8	< 8	< 8	8.4
157	< 8	< 8	< 8	3.7
165	< 8	< 8	< 8	5.3
166	< 8	< 8	< 8	11.5
167	< 8	< 8	< 8	4.6
168	20.2206	8.4014	33.5314	12.6

170	< 8	< 8	< 8	12.3
171	114.093	92.12	141.874	24.9
172	42.6382	33.947	52.11	9.1



Male Patient 149 no indication of malignancy or kidney stones
Male Patient 167 has kidney stones
Male Patient 165 has prostate cancer – post treatment
Male Patient 168 has prostate cancer – post treatment
Male Patient 170 has prostate cancer with a recent PSA of 6.6 -presurgery
-don't know the status of the other patients at this time.